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Running Title: BPA exposure and HPA-axis function in pregnancy

Urinary bisphenol A is associated with dysregulation of HPA-axis function in pregnant women: Findings from the APrON cohort study

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Abstract

Background: Bisphenol A (BPA) is associated with dysregulation of hypothalamic-pituitary-adrenal (HPA) axis activity in rodents, but evidence in humans is lacking.

Objective: To determine whether BPA exposure during pregnancy is associated with dysregulation of the HPA-axis, we examined the association between urinary BPA concentrations and diurnal salivary cortisol in pregnant women. Secondary analyses investigated whether the association between BPA and cortisol was dependent on fetal sex.

Methods: Diurnal salivary cortisol and urinary BPA were collected during pregnancy from 174 women in a longitudinal cohort study, the Alberta Pregnancy Outcomes and Nutrition (APrON) study. Associations between BPA and daytime cortisol and the cortisol awakening response (CAR) were estimated using mixed models after adjusting for covariates.

Results: Higher concentrations of total BPA uncorrected for urinary creatinine were associated with dysregulation of the daytime cortisol pattern, including reduced cortisol at waking, β=-.055, 95% CI (-.100, -.010) and a flatter daytime pattern, β=.014, 95% CI (.006, .022) and β=-.0007 95% CI (-.001, -.0002) for the linear and quadratic slopes, respectively. Effect sizes in creatinine corrected BPA models were slightly smaller. None of the interactions between fetal sex and BPA were significant (all 95% CI's include zero).

Conclusions: These findings provide the first human evidence suggesting that BPA exposure is associated with dysregulation of HPA-axis function during pregnancy.

Keywords: cortisol, pregnancy, bisphenol-A, HPA-axis function, cortisol awakening response

Introduction

Bisphenol A (BPA) is a synthetic chemical commonly used to produce a wide range of consumer products including epoxy resins, polycarbonate plastics, dental composites, thermal paper, and to line water pipes and many food and beverage cans (Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007). Biomonitoring studies conducted over the past 2 decades in Asia, Europe, the United States and Canada have detected BPA in the majority of individuals sampled (Bushnik et al., 2010; Health Canada, 2015; Vandenberg et al., 2010), and in 82% - 90% of pregnant women, with median concentrations between 0.82 – 2.6 ng/mL (Arbuckle et al., 2014; Braun et al., 2011; Braun et al., 2012; Chevrier et al., 2013; Snijder et al., 2013; Wolff et al., 2008).

The National Toxicology Program at the National Institute of Environmental Health Sciences conducted a review of potential adverse reproductive and developmental effects of BPA in 2008 and expressed concern regarding its potential effects on brain development and behavior in fetuses, infants and children (Chapin et al., 2008). Maternal BPA exposure in the perinatal period is a concern for fetal development because BPA can pass through the placenta (Balakrishnan, Henare, Thorstensen, Ponnampalam, & Mitchell, 2010) and cross the blood-brain barrier (Kim et al., 2004; Sun, Nakashima, Takahashi, Kuroda, & Nakashima, 2002).

BPA is an endocrine disrupting chemical (EDC) that interacts with many nuclear and non-nuclear receptors (Wetherill et al., 2007) and disrupts estrogen and thyroid neuroendocrine systems during development, including sexual differentiation of the brain (Chevrier et al., 2013; Palanza, Gioiosa, vom Saal, & Parmigiani, 2008; Palanza, Howdeshell, Parmigiani, & vom Saal, 2002; Palanza, Morellini, Parmigiani, & vom Saal, 1999; Palanza, Nagel, Parmigiani, & Vom Saal, 2016). Few studies have investigated the possibility that BPA disrupts the hypothalamic-

pituitary-adrenal (HPA) axis, another component within the neuroendocrine system. *In silico* (computer) modeling has shown that BPA can bind to glucocorticoid receptors as an agonist, making plausible the direct stimulation of the HPA-axis (Prasanth, Divya, & Sadasivan, 2010). *In vivo* animal models (primarily rodents) have shown that perinatal BPA exposure leads to increases in basal corticosterone and exaggerated corticosterone responses to a stressor in offspring (Chen, Zhou, Bai, Zhou, & Chen, 2014; Panagiotidou, Zerva, Mitsiou, Alexis, & Kitraki, 2014; Poimenova, Markaki, Rahiotis, & Kitraki, 2010). Sex differences were observed but were not consistent across studies, which could be related to factors such as dose and age of testing. Taken together, these findings support the hypotheses that BPA exposure alters HPA-axis activity and that fetal sex is an important effect modifier.

Dysregulation of the HPA-axis is commonly observed in children with behavioral problem (Doom & Gunnar, 2013) and may be a pathway by which perinatal BPA exposure is associated with anxiety- and depression-like behaviors in animals (Chen, Zhou, Bai, Zhou, & Chen, 2015) and behavioral disorders in children (Braun et al., 2011; Braun et al., 2009; Evans et al., 2014; Perera et al., 2012). During gestation, dysregulation of the maternal HPA-axis has broad and enduring effects on fetal development (Mina & Reynolds, 2014). Therefore, to the extent that BPA exposure alters maternal HPA-axis function, this represents a plausible mechanism by which BPA exposure may alter offspring behavior. Accordingly, the primary objective of the current study was to determine whether maternal urinary BPA was associated with dysregulation of maternal HPA-axis function during pregnancy. Given that most animal and human studies of BPA exposure have observed sex differences in biological and behavioral outcomes, and because it has recently been shown that maternal HPA-axis function during pregnancy differs as a function of fetal sex (DiPietro, Costigan, Kivlighan, Chen, &

Laudenslager, 2011; Giesbrecht, Campbell, & Letourneau, 2015), a secondary objective was to investigate whether the sex of the fetus alters the association between maternal BPA and HPA-axis function. To accomplish these aims, we measured diurnal salivary cortisol and urinary BPA in women during pregnancy.

Methods

Study population. Women were enrolled in the Alberta Pregnancy Outcomes and Nutrition (APrON) study between March 2009 and July 2012. APrON is a prospective longitudinal study that is following a community cohort of women and their offspring (n = 2140) who were recruited as early in pregnancy as possible, and in all cases prior to 27 weeks gestation. The sampling frame included all pregnant women over the age of 16 living within Alberta's two largest metropolitan areas, with a total population of approximately 2 million in in 2010. A complete description of the APrON study cohort and methods is available elsewhere (Kaplan et al., 2014; Leung et al., 2016). The current sample comprises 174 women who collected a 2nd trimester urine sample and who were enrolled in a sub study that collected saliva samples (for cortisol determination). The sample was enrolled between 6-22 weeks gestation; mean = 14.9 weeks gestation. A total of 309 women were approached to participate in the sub-study; of these 78 declined and 57 were excluded. Women were excluded from saliva collection if they smoked (n = 3) or consumed alcohol (n = 4) during pregnancy, had a non-singleton pregnancy (n = 9), had poor oral health (which may affect saliva sampling; n = 12), had significant pregnancy complications that would make diurnal saliva collection difficult (n = 10), or were taking a synthetic glucocorticoid medication (n =12) or an antidepressant (n = 7), both of which can alter HPA-axis function. The study protocol was approved by the University of Calgary Health Research Ethics Board and participants provided informed consent prior to data collection.

Procedures.

Salivary cortisol. Diurnal HPA-axis function can be non-invasively assessed via salivary cortisol. Participants collected diurnal saliva at two time points in pregnancy: T1 = 14-26 weeks gestation, and T2 = 32-36 weeks gestation. At each of these time points, women collected saliva on at least 1 and up to 3 regular weekdays on the following schedule: upon waking, 30 minutes after waking, at ~1130h, and at ~2030h. These time points were selected to capture the two main elements of the diurnal pattern: the cortisol awakening response (CAR), which refers to the surge in cortisol secretion that occurs within the first 30 minutes after waking, and the diurnal slope, which refers to the expected decline in cortisol from waking to the end of the day. Data collection was supported by a Personal Digital Assistant (PDA, i.e. Palm^{TM}), which rang each time that a sample was to be collected. On each sampling day, participants collected the waking sample as soon after waking as practically possible and then initiated a 30-minute timer on the PDA. This procedure allowed for precise timing of the waking plus 30-minute sample while also allowing for individual differences in waking times.

Each time the PDA rang, it first provided a code corresponding to a pre-labeled saliva tube and instructed the participant to place the saliva roll (Salivabio Oral Swab, Carlsbad, CA) under her tongue. The time of each sample collection was recorded by the PDA, and women recorded sample time in a sample diary – any time discrepancies between the PDA and diary were resolved through discussion with the participant. To facilitate adherence to the study protocol, the PDA was programmed to allow a 20-minute response window following the signal, after which data were considered missing.

Participants were asked to refrain from consuming food, caffeine, citric drinks and dairy, to avoid vigorous exercise (e.g., running) or brushing teeth in the 30 minutes prior to saliva sample

collection (because of potential effects on cortisol concentration), and to report adherence to these guidelines. Whole saliva was obtained from under the tongue. Saliva samples were temporarily stored in participants' home freezers (1-2 days) until they could be transported on ice packs to the laboratory. Samples were stored at -80° C until they were shipped frozen to Salimetrics (State College, PA).

All samples were assayed for salivary cortisol without modification to the manufacturer's instructions. The cortisol test has a lower limit of sensitivity of 0.007 µg/dL, standard curve range from 0.012 to 3.0 µg/dL, and average intra-and inter-assay coefficients of variation 3.5% and 5.1% respectively. Method accuracy, determined by spike and recovery, and linearity, determined by serial dilution are 100.8% and 91.7%, respectively. In the current study, 15% of samples were randomly selected and assayed in duplicate; the intra-assay coefficient of variation was low (4.9%) confirming reliability of the method. The mean value from duplicate assays was used in data analysis.

Urinary BPA. A spot urine sample was collected from each participant at T2 (14-26 weeks of gestation). Sterile urine cups were used to collect urine samples, which were immediately aliquoted into 9 mL cryovials and stored at -80 °C. Potential contamination of BPA from sampling and storage materials was tested by simulating urinary collection with clean liquid chromatography (LC) grade water (n = 20 times). Specifically, LC water was poured into the polypropylene sterile urine cups and transferred to 9 mL cryovials using the same type of kits used for real urine samples. No BPA was detected in these control samples.

Urinary concentrations of total BPA were quantified by online solid-phase extraction (online SPE) coupled to high performance liquid chromatography (HPLC) and an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). For each sample, 400 µL of urine

was mixed with 10 μL of 1 ng $^{13}C_{12}$-BPA, 200 μL of 1 M ammonium acetate buffer containing 1μL β-glucuronidase and sulfatase enzyme (Sigma, St. Louis, MO). The mixture was incubated at 37 °C overnight to deconjugate BPA metabolites back to free BPA, and then 390 μL of 1 M formic acid was added to bring the final volume to 1mL. For online SPE and HPLC, the mobile phases were both: (A) water and (B) methanol. 100 μL sample solvent (containing 40 μL of urine) was loaded on the Fisher Scientific Hypersil GOLD C18 column (12 μm, 20 mm × 2.1 mm) by a flow (2 mL/min) of 10% B for 1 min. The analytes were eluted by 0.5 mL/min 50% B to a Phenomenex Luna C18 column (3 μm, 4.60 mm × 150 mm) for chromatographic separation. The gradient program started at 0.8 mL/min 10% B, which changed to 50% B when the online SPE was eluted to the analytical column. After 5 min, the gradient ramped to 95% B in 1.5 min, held for 2 min, and then returned to initial condition in 3.5 min. A 3 min re-equilibration period was used before the next automatic sample injection.

The orbitrap mass spectrometer was operated in negative ionization mode using atmospheric pressure chemical ionization. Tandem mass spectrometry (MS/MS) scan mode was used for BPA and $^{13}C_{12}$-BPA (internal standard) detection. The recoveries of BPA at two levels (1 ng/mL, 10 ng/mL) were 88% and 102%, with relative standard deviations (RSD) of 11%. Linearity was evaluated over two orders of magnitude (0.5 ng/mL to 50 ng/mL, 6 point curve), and the regression coefficients of the standard curves were always > 0.99. The limit of detection was 0.32 ng/mL; BPA concentrations below the limit of detection were reported as the lower limit/1.414 for statistical analyses.

Urinary creatinine. A Synchron LX® systems (Beckman Coulter) was used to quantify urinary creatinine. The limit of detection of creatinine was 10 mg/dL, with a dynamic range from 18 to 399 mg/dL (r>0.99). The RSD of duplicate injections (n = 48) of one urine sample over 4 months

was 2%. Results for BPA concentration uncorrected for urinary creatinine and BPA adjusted for creatinine are reported in the statistical analysis. Statistical models were adjusted for creatinine in two different ways to reflect current practice (Barr et al., 2005): one was to add creatinine as an independent variable to the model (creatinine as covariate) and the other was to divide BPA by creatinine concentration (creatinine-corrected).

Potential covariates and confounders. Potential covariates for inclusion in statistical models had known associations with either urinary BPA, diurnal salivary cortisol, or both (Egliston, McMahon, & Austin, 2007; LaKind & Naiman, 2015). From maternal self-reports at the first study visit, we obtained measures of maternal age, income, pre-pregnancy body mass index (BMI), parity, education, and ethnicity. At each study visit, women were asked to report any medication use, and we obtained measures of depression and anxiety symptoms using well-validated measures during pregnancy. Depression symptoms were assessed via the Edinburgh Depression Scale (EDS), a 10-item instrument that is widely used to screen for perinatal depression (Cox, Holden, & Sagovsky, 1987; Matthey, 2016). Pregnancy anxiety symptoms were assessed using the Pregnancy Anxiety Scale (PAS), a 10-item self-report instrument that quantifies the extent to which pregnant women worry about their health, their baby's health, labor and delivery, and caring for their baby (Rini, Dunkel-Schetter, Wadhwa, & Sandman, 1999).

Statistical analysis. Linear growth curve models were estimated using the MIXED procedure in SPSS version 22.0 in order to determine if urinary BPA concentration was associated with the diurnal salivary cortisol patterns. The normal diurnal pattern for cortisol begins at waking with high concentrations, which then decline over the course of the day (referred to as the daytime slope). The daytime slope is typically quantified using a variable for time-since-waking and a

variable for time-since-waking squared (to account for the deceleration of decline toward the end of the day). Superimposed on this overall daytime decline is a sharp concentration increase during the first 30-45 minutes post waking (referred to as the cortisol awakening response: CAR). Because previous studies have shown that the daytime slope and CAR are distinct elements of the diurnal pattern (Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010; Wilhelm, Born, Kudielka, Schlotz, & Wust, 2007), separate models were estimated for the CAR and daytime slops. Prior to analyses, data were screened to ensure sample time adherence because valid assessment of the CAR requires sample collection within acceptable time windows. As recommended (Okun et al., 2010), waking samples were excluded if they were collected more than 10 minutes after waking and samples collected at 30 minutes post waking were excluded if they were collected more than 49 minutes after waking. Missing values were estimated using maximum likelihood. Given their right-skewed distribution, cortisol values were natural log transformed prior to data analysis.

Multilevel equations were specified at two levels to account for the nesting of repeated cortisol measures within each person. The outcome for all models was natural log transformed cortisol. The daytime slope model included time-since-waking (in hours) and time-since-waking squared to capture the curvilinear change in cortisol over the day. Time was centered at waking so that the intercept reflected waking levels. At level 2, the focal predictors were BPA (log10 transformed and grand mean centered) and the cross-level interactions between BPA and time. In the daytime slope model, the parameter estimates for BPA describe the association between BPA and the diurnal cortisol pattern. The models for the CAR were the same as those for the daytime slope model with the exception that time-since-waking squared was not included in the model, and time was centered at 30 minutes post waking so that the parameter estimate for BPA

describes differences in cortisol at 30 min post waking as a function of BPA. To select covariates for inclusion in the final models, we assessed the association of potential covariates with diurnal cortisol in preliminary models (using $p < .10$ as criterion for retention) and using a likelihood ratio test to evaluate model fit (using $p < .05$ as criterion). Potential covariates that were eliminated from further consideration included medication use, maternal age, income, pre-pregnancy body mass index (BMI), education, anxiety symptoms, and depression symptoms; those that were retained included parity and ethnicity (dichotomized to White/Other[1]). Time of urine sample collection was included in all models to account for diurnal variation in urinary BPA in pregnant women (Braun et al., 2011). Gestational age at each study assessment was also included in each model to account for the normal increases in cortisol over the course of gestation.

Secondary Analyses

To determine whether the association between BPA and cortisol was moderated by fetal sex, a dummy variable for sex (males = 0) was added to the above models, along with interaction terms between BPA and fetal sex. To determine the extent to which urinary dilution/concentration may affect the association between BPA and cortisol, all analyses were re-run excluding women who had urinary creatinine concentrations that were considered too diluted (2.653 mmol/L) or too concentrated (26.53mmol/L) according to the World Health Organization guidelines for occupational monitoring, which have also been applied to non-occupational studies (Barr et al., 2005).

Results

[1] Other includes Hispanic Whites.

Overall, the sample was relatively well-educated (71% had a university education), married or living in common-law relationships (99%), mature (geometric mean age = 31.5 years, range 22.4-42.8 years) and nulliparous (53%). The majority of women (86%) lived in households with annual income greater than $70,000 CAD (according to Statistics Canada the median household income within the recruitment region was $98,030), and most were White (87%). As the sample was embedded within a larger cohort study, we tested for potential selection bias by comparing the characteristics of women included in this analyses and the full APrON cohort. Women in the current analysis had more education, (71% completed a university degree versus 68% in the full cohort) and had higher pre-pregnancy BMI (25.5 kg/m^2 versus 24.1 kg/m^2). The sample did not differ from the full cohort on income, ethnicity, age, parity, or marital status (all p values > .05).

Characteristics of the sample by BPA tertile are listed in Table 1 and by cortisol tertile in Table 2. Of note, Table 1 reveals that T1 and T2 cortisol concentrations did not differ by BPA tertile, $F(2, 171) = .23$, $p = .80$ and $F(2, 171) = .42$, $p = .66$, respectively. However, creatinine concentrations increased by BPA tertile, $F(2, 171) = 36.36$, $p < .001$. Table 2 shows that total BPA concentrations were somewhat lower in the upper tertile of cortisol, compared to those in the lower and middle tertiles, however group differences were not statistically significant, $F(2,171) = 1.78$, $p = .17$. Likewise, there was no difference in creatinine by cortisol tertile, $F(2,171) = 1.27$, $p = .28$.

BPA concentrations above the lower limit of detection were observed in 91.4% of the samples. Average BPA concentrations (GM = 1.11 ng/mL, range .16 – 43.2 ng/mL) were within the range previously reported for pregnant women in North America. Creatinine was detected in all samples. Cortisol was detected in all but one sample, and one cortisol value was not

biologically plausible (> 7 µg/dL); both values were removed. As expected, salivary cortisol concentrations displayed a diurnal pattern over the course of the day (see Figures 1 and 2), with a steep increase in the first 30 minutes post waking and a gradual decline over the remainder of the day, and overall concentrations increased with gestational age, GM = .32 and .41 µg/dL for 2nd and 3rd trimester, respectively. A total of 2819 cortisol and 174 BPA samples were available for analysis.

Associations between BPA and daytime cortisol

Statistically significant associations were observed between BPA uncorrected for urinary creatinine and all three parameters of the diurnal cortisol pattern (i.e., intercept, linear slope and quadratic slope). Specifically, for each 10-fold increase in BPA, waking cortisol levels were 5.4% lower, the linear slope was more positive (flatter by 1.4% per hour), and the quadratic slope more negative (flatter by .07% per hour^2), see Table 3, Model 1. Given that we observed an 18.4-fold change in BPA concentration between the mean of the lower and upper quartiles, these model estimates indicate a potential for a 10% decrease in cortisol at waking, a 2.6% flatter linear slope and a .1% flatter quadratic slope for the average woman in the upper compared to the lower BPA quartile. To illustrate the association between BPA and daytime cortisol, the estimates obtained in Model 1 have been graphed at the mean of the upper and lower quartiles for BPA to represent the highest and lowest exposures in the sample. Effects sizes were slightly smaller when creatinine was added as an independent variable to the model (see Table 3, Model 2) and the confidence interval for the intercept included the null value. Otherwise, Models 1 and 2 had the same findings. Creatinine correction further reduced the effect sizes and the only confidence internal that did not contain the null value was the association for the linear slope; the

estimate for this association was a 1% flatter slope per hour for each 10-fold increase in BPA (see Table 3, Model 3).

Associations between BPA and cortisol awakening response (CAR)

All confidence intervals for associations between BPA and the CAR included the null value (see Table 4, Models 1-3). The effect size for the association between BPA and cortisol at 30 minutes post waking was similar to that observed at waking (4% - 5% decrease, see Table 4), however, as shown in Figure 2, the slope for the awakening response did not differ as a function of BPA exposure and thus individuals with higher BPA had lower cortisol at both waking and 30-45 minutes post waking.

Secondary Analyses

Confidence intervals for the interaction term between fetal sex and BPA included zero in all models (results not shown), indicating that the association between BPA and cortisol was not dependent on fetal sex.

To determine whether urinary dilution/concentration may have affected the results, we excluded women with low (n = 18) or high (n = 1) creatinine concentrations (using the WHO criteria defined above). Parameter estimates for the association between BPA and cortisol were somewhat attenuated in all models (results not shown). In the uncorrected BPA model, the associations between BPA and daytime cortisol remained the same as those reported in Table 3, Model 1. For models in which creatinine was added as a covariate or BPA was creatinine corrected, all estimates for the associations between BPA and cortisol included the null value. All associations between BPA and cortisol in the CAR models remained non-significant after exclusion for urinary dilution/concentration.

Discussion

The primary finding of this study was that higher levels of urinary BPA were associated with reduced waking levels and flattening of the diurnal cortisol patterns in pregnant women. The findings for models in which creatinine was added as in independent variable or in which BPA was corrected for creatinine were similar to those for uncorrected BPA concentrations, although the effect sizes were slightly reduced. Associations between BPA and HPA-axis function did not differ as a function of fetal sex. These findings provide the first human evidence suggesting that dysregulation of HPA-axis function is associated with higher exposure to BPA.

Urinary BPA concentrations above the lower limit were detected in 91% of the sample, similar to the 82% - 90% detected in other samples of pregnant women (Arbuckle et al., 2014; Braun et al., 2011; Braun et al., 2012; Chevrier et al., 2013; Wolff et al., 2008). Uncorrected urinary BPA concentrations in the current sample (1.11 ng/mL) were higher compared to pregnant women in the MIREC (.80 ng/mL) (Arbuckle et al., 2014) and CHAMACOS (1.0 ng/mL) (Harley et al., 2013) cohorts, and lower than pregnant Dutch women in the Generation R study (1.3 ng/mL) (Snijder et al., 2013), and women of childbearing age in the Canadian population (1.26 ng/mL) (Bushnik et al., 2010).

Previous studies assessing the effects of BPA on HPA-axis functioning are limited to in-silico models and animal models. The in-silico study suggested that BPA may bind to the glucocorticoid receptor as an agonist and thereby induce biological effects similar to those produced by glucocorticoids (Prasanth et al., 2010). Numerous rodent studies show that perinatal exposure to BPA results in dysregulation of the offspring HPA-axis (Chen et al., 2014; Panagiotidou et al., 2014; Poimenova et al., 2010), although it should be noted that the exposures in these studies, even the 'low-dose' studies, are orders of magnitude above the median daily intake estimates for adults in the US (LaKind & Naiman, 2015). The current findings are

consistent with animal evidence of HPA-axis disruption associated with BPA exposure and extend these findings to the pregnant mother.

The observed associations between BPA and daytime cortisol resemble previous reports of the associations between depression and daytime cortisol. For example, Jarcho and colleagues (2013) reported that depressed women had lower waking cortisol levels and flatter diurnal cortisol rhythms compared with non-depressed women, but the cortisol awakening response was not affected (Jarcho, Slavich, Tylova-Stein, Wolkowitz, & Burke, 2013). Although this pattern is similar to those observed in the current study for women with elevated BPA exposure, it should be noted that maternal depression and anxiety were eliminated from statistical models because they were not associated with the diurnal pattern in the present study. As a result, the similarities in the daytime cortisol pattern observed here in relation to BPA and those observed elsewhere in relation to depression may be coincidental. Nevertheless, the possibility that BPA exposures contribute to depression via dysregulation of the HPA axis should be evaluated in future studies.

Our findings provide preliminary evidence that BPA is associated with dysregulation of HPA-axis function in humans, yet the biological mechanisms that underlie these effects remain unknown. It may be possible to gain new mechanistic insights into the effects of BPA on the HPA-axis by administering dexamethasone to non-pregnant humans or animals with varying BPA exposures. Flattening of the daytime cortisol pattern may be due, at least in part, to glucocorticoid resistance and impaired glucocorticoid sensitivity in the paraventricular nucleus of the hypothalamus and the pituitary gland (Jarcho et al., 2013), which can be assessed via administering dexamethasone. Such research will provide further insight into the potential role of BPA in HPA-axis functioning and also to increase confidence that the associations observed here are direct and not due to some other associated factor.

Although fetal sex is associated with changes in patterns of cortisol secretion in pregnant women (DiPietro et al., 2011; Giesbrecht et al., 2015), there was no evidence that the association between BPA and maternal cortisol was moderated by fetal sex. Nevertheless, it is important to note that the current sample size was modest, and given the potential implications for child neurodevelopment and behaviour, the possibility of interaction effects should be examined in a larger cohort.

Urinary dilution/concentrations, as estimated by creatinine, altered the associations between BPA and cortisol. Inclusion of creatinine in the models, whether as an independent variable, a correction factor, or an exclusion criterion reduced the observed effect sizes. However, reliable associations were observed after each form of adjustment for creatinine, suggesting that the association between BPA and daytime cortisol cannot be accounted for by urinary dilution/concentration.

Strengths and limitations

This study had several strengths. We used the most reliable analytical methods to measure BPA and cortisol concentrations in appropriate samples and included covariates identified in previous research. Thus, the possibility of measurement bias is decreased. These data are novel and timely given the continued interest in endocrine disrupting chemicals and the lack of epidemiological (or animal) studies exploring their impact on HPA-axis function.

This study also had several limitations. First, considering that our analysis was restricted to the subset of women from our larger sample who collected both urine and saliva samples, it is possible that our results were subject to selection bias. We tested for potential selection bias by comparing the sociodemographics of women included in the sample to those in the larger cohort. Women in our sample had more education, higher pre-pregnancy BMI, and there was a higher

proportion of White women in our sample compared to the full cohort, but the groups did not differ on income, age, parity, or marital status. It should be noted that the saliva sampling procedures used in this study incur a fairly high degree of participant burden and this may have contributed to the composition of the sub-study sample. Considering that the full APrON cohort is more White, educated, has higher household income, and is older compared to child bearing women in Canada as a whole (Leung, McDonald, Kaplan, Giesbrecht, & Tough, 2013), generalization to other ethnic and socioeconomic populations should be made with caution. Second, the use of one measure of BPA exposure in mid-pregnancy assumes that this measurement is representative of exposure during pregnancy. BPA has relatively short half-life (Taylor et al., 2011) and there is inherent variability in BPA concentrations over time, which pregnancy may accentuate (Braun et al., 2011). Accordingly, a single spot urine sample may not accurately reflect BPA exposure over pregnancy. To reduce measurement variability, we included time of urine sample collection in our statistical models, as suggested by Braun and colleagues (Braun et al., 2011). Furthermore, potential measurement bias would most likely be non-differential, such that the error in measurement equally applies to both lower BPA concentrations and higher BPA concentrations. Consequently, any measurement bias would decrease the association between BPA urinary concentrations and maternal cortisol. Thus, the effects observed here are more likely to underestimate than to overestimate the true association between BPA and cortisol. Finally, although we tested many potential covariates and included those that were theoretically important and statistically related to the outcome in our models, the existence of residual confounding in our findings cannot be disregarded.

Conclusion

Despite multiple studies linking perinatal BPA exposure to offspring HPA-axis function in rodent models, to our knowledge this is the first study linking BPA concentration to human HPA-axis function. Our findings are consistent with the hypothesis that BPA disrupts HPA-axis function in pregnant women. Additionally, this study offers preliminary evidence that fetal sex does not modify the association between BPA and HPA-axis function in pregnant women. Given that dysregulation of the maternal HPA-axis is known to have broad and enduring effects on fetal development, further prospective research is needed to confirm the results of our study and to extend these findings to HPA-axis functioning in children.

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Table 1. Sample characteristics and descriptive statistics by BPA tertile.

	Lower tertile (n = 58)			**Middle tertile (n = 58)**			**Upper tertile (n = 58)**		
Maternal Variable	***GM(SD)***	***Median***	***Range***	***GM(SD)***	***Median***	***Range***	***GM(SD)***	***Median***	***Range***
Age (years)	30.6 (4.1)	30.7	23.1 – 41.0	31.9 (3.4)	31.9	24.5 – 39.2	31.9 (4.0)	32.4	22.4 – 42.8
Pre-pregnancy BMI	24.6 (4.6)	24.1	17.1 – 42.3	25.0 (5.5)	24.4	18.0 – 46.9	25.1 (6.3)	23.9	18.5 – 44.9
Total BPA (ng/mL)	.36 (.14)	.35	.16 - .66	1.02 (.29)	1.00	.66 – 1.60	3.82 (7.99)	3.22	1.66 – 43.20
Creatinine (mmol/L)	3.86 (3.05)	3.85	.70 – 13.0	7.93 (4.96)	9.15	1.80 – 19.10	11.07 (6.52)	11.25	1.70 – 32.80
BPA corrected for Creatinine (μg/g)	.82 (.79)	.77	.24 – 4.00	1.13 (.99)	1.02	.36 – 4.58	3.05 (8.88)	2.89	.76 – 59.67
2nd trimester cortisol (μg/dL)	.33 (.08)	.34	.21 - .66	.32 (.10)	.32	.17 – .78	.31 (.11)	.32	.12 – .71
3rd trimester cortisol (μg/dL)	.42 (.19)	.42	.24 - .85	.41 (.13)	.40	.23 – 1.00	.41 (.09)	.42	.22 – .60
	Percent			**Percent**			**Percent**		
Household Income									
<$20,000	3.6			1.7			0		
$20k – $40k	1.8			5.2			5.2		
$40k – $70k	7.3			8.6			8.6		
$70k – $100k	23.6			22.4			34.5		
>$100,000	63.8			62.1			51.7		
Education									
High school diploma	12.7			1.7			5.2		

College diploma	10.9	24.1	27.6
University degree	76.3	74.1	67.3
Ethnicity			
White	87.9	86.2	87.9
Other	12.1	13.8	12.1
Marital status			
Married	89.1	82.3	91.4
Common law	9.1	17.2	8.6
Single/separated/ divorced	1.8	0	0
Nulliparous	51.7	50.0	56.9

Note. GM = geometric mean

Table 2. Sample characteristics and descriptive statistics by Cortisol tertile.

	Lower tertile (n = 58)			**Middle tertile (n = 58)**			**Upper tertile (n = 58)**		
Maternal Variable	***GM(SD)***	***Median***	***Range***	***GM(SD)***	***Median***	***Range***	***GM(SD)***	***Median***	***Range***
Age (years)	31.9 (3.9)	32.0	24.4 – 41.0	31.0 (3.8)	32.1	22.4 – 38.7	31.6 (3.9)	31.4	25.7 – 42.8
Pre-pregnancy BMI	24.9 (5.9)	24.1	18.5 – 44.9	24.5 (4.9)	23.6	18.0- 43.1	25.3 (5.7)	24.4	17.1 – 46.9
Total BPA (ng/mL)	1.20 (6.35)	.97	.23 – 39.84	1.33 (5.97)	1.19	.23 – 43.20	.87 (1.79)	.91	.16 – 11.07
Creatinine (mmol/L)	6.73 (6.12)	6.90	.70-25.30	7.76 (6.51)	8.65	.90 – 32.80	6.51 (5.24)	7.00	.80 – 21.10
BPA corrected for Creatinine (μg/g)	1.57 (4.36)	1.50	.28 – 25.21	1.51 (8.24)	1.14	.24 – 59.67	1.18 (2.30)	1.07	.27 – 16.59
2nd trimester cortisol (μg/dL)	.27 (.07)	.27	.12 – .39	.34 (.11)	.32	.22 – .71	.38 (.09)	.38	.26 – .78
3rd trimester cortisol (μg/dL)	.31 (.04)	.32	.22 – .36	.41 (.03)	.41	.36 – .47	.55 (.10)	.53	.47 – 1.00
	Percent			**Percent**			**Percent**		
Household Income									
<$20,000	3.3			1.9			0		
$20k – $40k	0			3.7			8.9		
$40k – $70k	9.8			5.6			8.9		
$70k – $100k	34.4			25.9			19.6		
>$100,000	52.5			63.0			62.5		
Education									
High school diploma	6.6			9.3			3.46		

College diploma	21.3	22.2	19.6
University degree	72.1	68.5	76.8
Ethnicity			
White	90.2	85.7	86.0
Other	9.8	14.3	14.0
Marital status			
Married	91.8	83.3	87.5
Common law	6.6	16.7	12.5
Single/separated/ divorced	1.6	0	0
Nulliparous	52.5	51.8	54.4

Note. GM = geometric mean.

Table 3. Model estimates for the effects of BPA on the daytime cortisol pattern.

	Model 1: BPA uncorrected for urinary dilution		Model 2: BPA with creatinine as covariate		Model 3: BPA creatinine-corrected	
Fixed Effects	β Coefficient (95% CI)	% difference (95% CI)	β Coefficient (95% CI)	% difference (95% CI)	β Coefficient (95% CI)	% difference (95% CI)
INTERCEPT	.359 (.324, .393)	GM = .43 μg/dL at waking	.362 (.319, .404)	GM = .44 μg/dL at waking	.360 (.325, .395)	GM = .38 μg/dL at waking
Gestational Age	.002 (.001, .003)		.002 (.001, .003)		.002 (.001, .003)	
Ethnicity	-.005 (-.043, .034)		-.006 (-.045, .034)		-.005 (-.044, .034)	
Parity	.013 (-.003, .028)		.013 (-.003, .029)		.012 (-.004, .028)	
Urine sample time	.002 (-.000003, .004)		.002 (-.00001, .004)		.002 (-.00001, .004)	
Creatinine			-.0004 (-.003, .002)			
BPA	**-.055 (-.100, -.010)**	-5.4% (-10, -1) at waking	**-.051 (-.103, .0008)**	-5.0% (-10, .08) at waking	**-.040 (-.090, .009)**	-4% (-9, .9) at waking
TIME (hours)	-.044 (-.047, -.040)	-4.3% (-5, -4) per hour	-.045 (-.050, -.040)	-4.5% (-5, -4) per hour	-.044 (-.047, -.041)	-4.3% (-5, -4) per hour
Gestational Age	.001 (.0006, .001)		.001 (.0006, .001)		.0009 (.0006, .001)	
Ethnicity	.008 (.0002, .015)		.008 (.0004, .016)		.008 (.0004, .016)	
Parity	-.002 (-.005, .0003)		-.002 (-.005, .0003)		-.002 (-.005, .0005)	

Creatinine			.0002 (-.0003, .0006)			
BPA	**.014 (.006, .022)**	1.4% (.6, 2) per hour	**.012 (.003, .022)**	1.2% (.3, 2) per hour	**.010 (.001, .019)**	1.0% (-.01, 2) per hour
$TIME^2$ (hours)	.002 (.001, .002)	.2% (.1, .2) per $hour^2$	.002 (.001, .002)	.2% (.1, .2) per $hour^2$	.002 (.002, .002)	.2% (.1, .2) per $hour^2$
Gestational Age	-.00005 (-.00007, -.00004)		-.00005 (-.00007, -.00004)		.0009 (-.001, .0003)	
Ethnicity	-.0005 (-.001, -.0006)		-.0005 (-.001, -.0001)		-.0005 (-.001, -.0001)	
Parity	.0001 (-.00001, .0003)		.0001 (.00007, .0003)		.0001 (-.0001, .0003)	
Creatinine			-.00001 (-.00004, .00002)			
BPA	**-.0007 (-.001, -.0002)**	-.07% (-.1, -.02) per $hour^2$	**-.0006 (-.001, -.00003)**	-.06% (-.1, -.003) per $hour^2$	**-.0005 (-.001, .0001)**	-.05% (-.10, .01) per $hour^2$

Note. Time was centered at waking; BPA is centered at the grand mean. Percent increase for BPA estimates are per 10-fold increase in BPA.

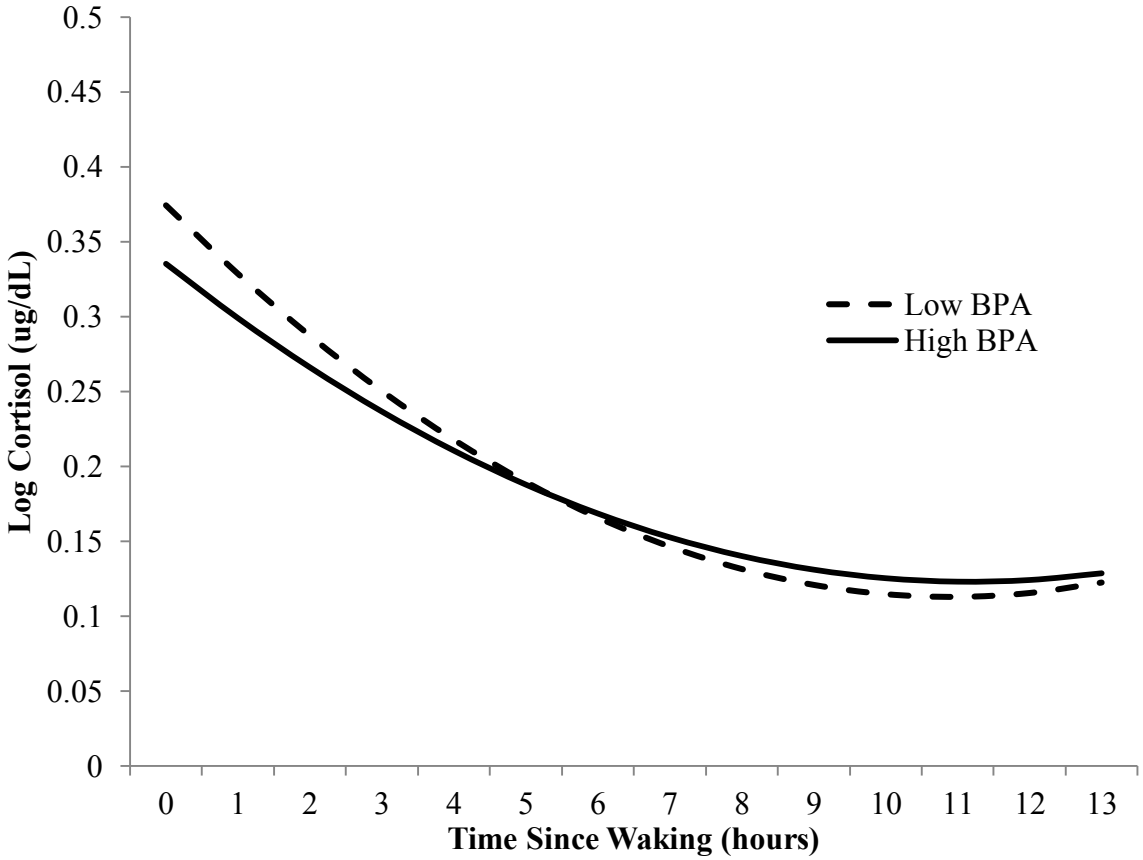
Table 4. Model estimates for effects of BPA and fetal sex on the cortisol awakening response.

	Model 1: BPA uncorrected for urinary dilution		Model 2: BPA with creatinine as covariate		Model 3: Creatinine-Corrected BPA	
Fixed Effects	β Coefficient (95% CI)	% difference (95% CI)	β Coefficient (95% CI)	% difference (95% CI)	β Coefficient (95% CI)	% difference (95% CI)
INTERCEPT	.453 (.364, .542)	GM = .57 µg/dL at 30-45 min post waking	.449 (.363, .544)	GM = .57 µg/dL at 30-45 min post waking	.443 (.357, .529)	GM = .56 µg/dL at 30-45 min post waking
Gestational Age	.003 (.002, .004)		.004 (.002, .004)		.003 (.002, .004)	
Ethnicity	-.003 (-.049, .042)		-.003 (-.049, .043)		-.003 (-.049, .043)	
Parity	-.0002 (-.019, .019)		-.0004 (-.020, .019)		-.001 (-.020, .018)	
Urine sample time	-.0002 (-.006, .005)		-.0002 (-.006, .005)		-.0003 (-.006, .005)	
Creatinine			.0003 (-.003, .003)			
BPA	**-.047 (-.103, .008)**	**- 5% (-10, .8)**	**-.051 (-.115, .013)**	**-5% (-11, 1)**	**-.041 (-.097, .014)**	**-4% (-9, 1)**
TIME (hours)	.153 (.123, .183)	17% (13, 20) per hour	.133 (.086, .179)	14% (9, 20) per hour	.149 (.119, .178)	16% (13, 19) per hour
Gestational Age	.006 (.003, .009)		.006 (.003, .009)		.006 (.003, .009)	
Ethnicity	.061 (-.007, .128)		.063 (-.004, .131)		.062 (-.006, .130)	
Parity	-.037 (-.061, -.012)		-.038 (-.062, -.013)		-.037 (-.062, -.012)	
Creatinine			.002 (-.002, .006)			

BPA	**-.017 (-.091, .057)**	-2% (-9, 6) per hour	**-.037 (-.120, .046)**	-4% (-12, 5) per hour	**-.033 (-.105, .040)**	-3% (-10, 4) per hour

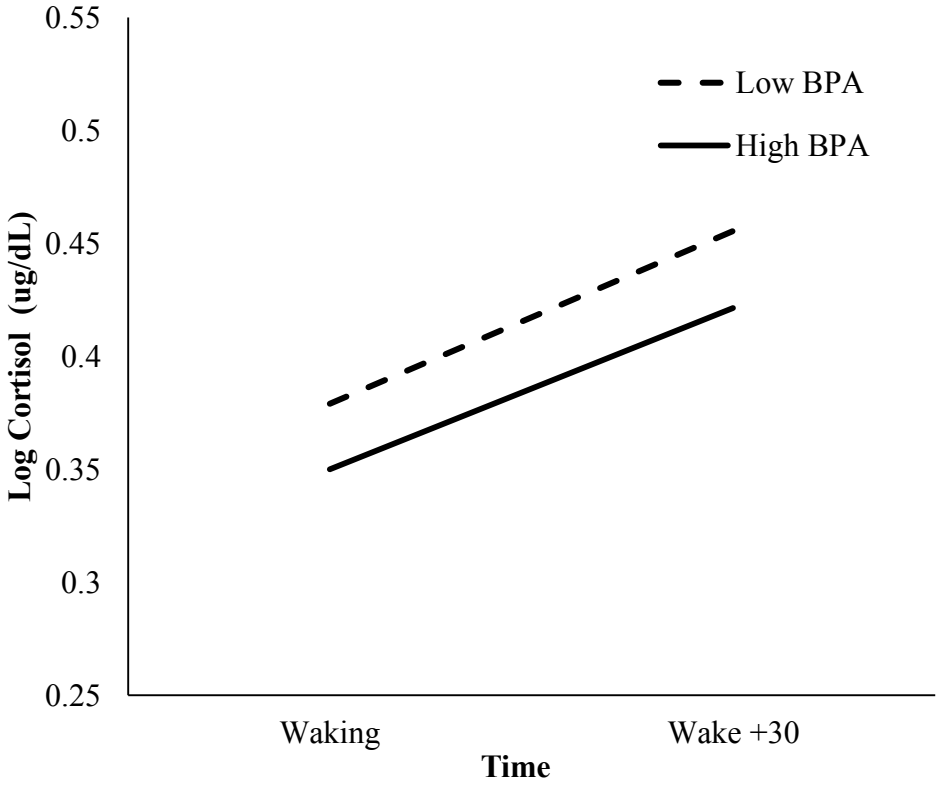
Note. Time was centered at 30 minutes post waking; BPA was centered at the grand mean. Percent increase for BPA estimates are per 10-fold increase in BPA.

Figure 1. Daytime cortisol as a function of time since waking for "Low" (lowest quartile) and "High" (highest quartile) maternal BPA



Note. All variables were modeled as continuous, however for the purpose of illustration the associations are displayed at the mean of the lower and upper quartiles to indicate the range of effects observed. Significant differences were observed for waking (time = 0), the linear slope, and the quadratic slope.

Figure 2. Cortisol awakening response (CAR) "Low" (lowest quartile) and "High" (highest quartile) maternal BPA



Note. All variables were modeled as continuous, however for the purpose of illustration the associations are displayed at the mean of the lower and upper quartiles to indicate the range of effects observed. There were no significant associations between BPA and the CAR.

Declarations

Ethics approval and consent to participate

This work was conducted in accordance with procedures approved by the University of Calgary Conjoint Health Research Ethics Board for use of human subjects in research. Participation of subjects did not occur until after informed consent was obtained.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

All authors contributed to the conceptualization of this study, contributed to revising the manuscript, and approved the final manuscript. GFG conducted the data analyses; JL

performed the laboratory analysis of BPA; GFG, JL, and ME composed the first draft of the manuscript.

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